ON THE AGGREGATION OF BOVINE SERUM ALBUMIN IN ACID *p*H

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The effects of low and high *p*H on the state of aggregation of bovine serum albumin have been the subject of a number of recent publications.[3-5] It is the purpose of this communication to report on some light scattering and ultracentrifugal measurements carried out on crystallized Armour bovine plasma albumin (BSA) in the *p*H range of 3 to 5 as a function of ionic strength, *p*H, preliminary deionization of the protein solution,[6] and length of time that the protein solution was permitted to remain at room and refrigerator temperatures.

The light scattering data obtained as a function of angle were measured on an instrument whose calibration was described in a recent publication,[7] while the light scattering experiments carried out as a function of concentration at 90° were performed in the previously used photometer[8,9] with a 3-ml. cell[10] on solutions cleared for light scattering as previously described.[11] The ultracentrifugal measurements were carried out at room temperature in a Spinco Model E analytical ultracentrifuge at 59,780 r.p.m.

The results of the multiangle light scattering experiments as a function of time are shown in Figs. 1 and 2. The curves in Fig. 1 were obtained using non-deionized BSA in *p*H 4.0 acetate buffer of 0.20 ionic strength. Curves A and B were obtained with a 2.1% protein solution, A at 325 to 650 sec. after dilution, and B at 4,500 to 4,600 sec. This solution was then diluted in the cell with clean buffer and filtered. The light scattering results obtained with the resulting 0.78% protein solution are shown by curves C and D, obtained at 8,000 and 20,000 sec., respectively. While curves A and B indicate an increasing molecular weight[12] and dissymmetry with time, curves C and D show a disaggregation and disappearance of very large aggregates upon dilution as evidenced by the great reduction of dissymmetry in these measurements. Similar results were obtained with another non-deionized solution at *p*H 4.0 which, however, had not been filtered after dilution, as well as with a non-deionized BSA solution in *p*H 5.0 acetate buffer of 0.20 ionic strength.

When an experiment was carried out with deionized BSA in *p*H 4.5 acetate buffer of 0.20 ionic strength no change in turbidity or appearance of dissymmetry occurred upon standing for close to 4.5 hours at room temperature.

In Fig. 2 are presented the low *p*H multiangle light scattering results obtained with two deionized preparations of BSA. The stock solution of protein was deionized at room temperature and lyophilized prior to use. In the first experiment, shown by curves A and B of Fig. 2, the lyophilized protein was dissolved in a 0.20 *M* NaCl solution of *p*H 3.0 and permitted to stand overnight at refrigerator temperature. This was then brought to room temperature, filtered and diluted to the concentration of the measurement. The results indicate that a considerable degree of aggregation occurs with standing at room temperature. The angular envelope, however, shows little dissymmetry.[16] Thus, immediately after dilution, the molecular weight was 100,000. This value had increased to 111,000 after 3.5 hours of standing

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(3) M. E. Reichmann and P. A. Charlwood, *Can. J. Chem.*, **32**, 1092 (1954).

(4) H. A. Saroff, G. I. Loeb and H. A. Scheraga, *J. Am. Chem. Soc.*, **77**, 2908 (1955).

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(6) H. Dintzis, Doctoral Dissertation, Harvard University, 1952.

(7) M. J. Kronman and M. D. Stern, This Journal, **59**, 969 (1955).

(8) B. A. Brice, M. Halwer and R. Speiser, *J. Optical Soc. Am.*, **40**, 768 (1950).

(9) M. Halwer, G. C. Nutting and B. A. Brice, *J. Am. Chem. Soc.*, **73**, 2786 (1951).

(10) H. M. Dintzis, in press.

(11) F. F. Nord, M. Bier and S. N. Timasheff, *J. Am. Chem. Soc.*, **73**, 289 (1951).

(12) Since all multiangle light scattering measurements were taken at a single concentration, the assumption was made that the second virial coefficient is very small at an ionic strength of 0.20 and can be neglected. This assumption has been shown experimentally to be valid as a close approximation.[13] It was also assumed that the thermodynamic interaction term contribution to the intercept in multicomponent light scattering measurements[14] was negligible in the case of BSA.[15]

(13) R. J. Gibbs and S. N. Timasheff, to be submitted for publication to the *Arch. Biochem. and Biophys.*

(14) J. G. Kirkwood and R. J. Goldberg, *J. Chem. Phys.*, **18**, 54 (1950).

(15) H. M. Dintzis, S. N. Timasheff, J. G. Kirkwood and B. D. Coleman, Abstracts, 126th Meeting of the American Chemical Society, New York, Sept., 1954.

(16) If the solution was filtered and used immediately after adjustment of *p*H to 3.0, a large dissymmetry appeared. This was followed by the precipitation of the residual bound ketone[17] liberated from the protein.

(17) P. Bro, private communication to S. N. Timasheff.

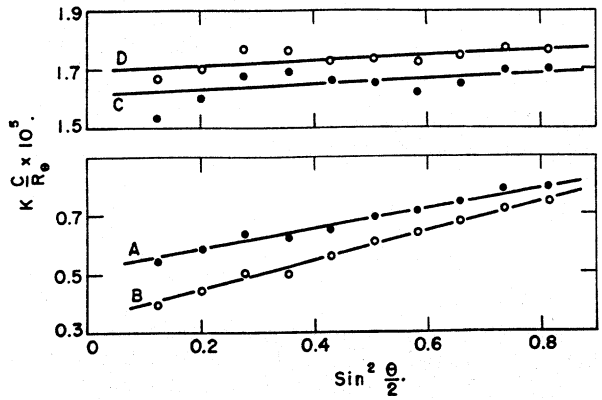

Fig. 1.—Angular scattering envelope for non-deionized BSA solutions in *p*H 4.0 acetate buffer ($\Gamma/2 = 0.20$): A, 2.1% protein, 325–650 sec; B, same, 4,500–4,600 sec.; C, solution diluted to 0.78%, 8,000 sec.; D, same, 20,000 sec.

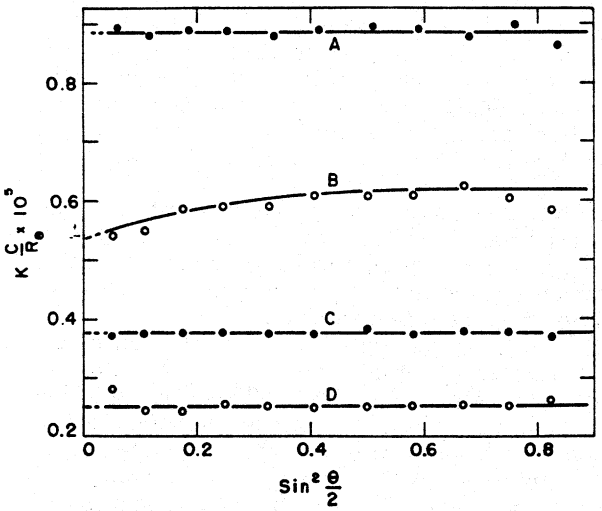

Fig. 2.—Angular scattering envelope for deionized BSA solutions in *p*H 3.0 HCl–NaCl (0.20 *M* NaCl): A, 0.72% protein, 13,000–14,000 sec.; B, same, 75,300–76,100 sec.; C, 0.68% protein, 100–800 sec.; D, same, 62,000–63,000 sec. (All times are from the moment of dilution. The concentrated stock solutions had remained overnight at refrigerator temperature.)

and to 182,000 after 21 hours. Ultracentrifugal analysis[18] of this solution at the end of the light scattering experiment showed the presence of two heavy components (10% of $S_{20,w} = 4.55$, 15% of $S_{20,w} = 8.97$) in addition to the normal albumin boundary ($S_{20,w} = 3.18$) (Fig. 3a). Such a composition of the solution corresponds well to a weight average molecular weight of 182,000.

Another run done under similar conditions and shown by curves C and D of Fig. 2, indicates that a protein solution which had aggregated to a molecular weight of 400,000 after standing for 20 hours after dilution at room temperature (curve D) displayed no angular dissymmetry. This would indicate that extensive aggregation can occur in BSA at *p*H 3.0 without increasing drastically the length of the kinetic unit. Such a case would be possible

(18) All values of sedimentation constants and area analysis are those measured at a total protein concentration of 0.7–0.8%. They are not corrected for the Johnston and Ogston anomaly.[19]

(19) J. P. Johnston and A. G. Ogston, *Trans. Faraday Soc.*, **42**, 789 (1946.)

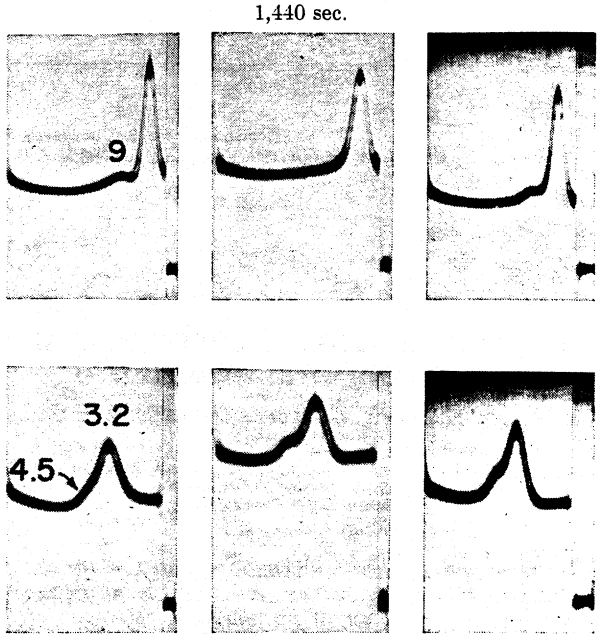

Fig. 3.—Ultracentrifugal patterns of deionized BSA in *p*H 3.0 HCl–KCl ($\Gamma/2 = 0.20$): a, solution deionized at 25° and lyophilized; run immediately after light scattering (curve B of Fig. 2); b, solution deionized at 4° and used fresh; analyzed in ultracentrifuge after standing 5 days at 25° and 21 days at 4° at *p*H 3.0; c, solution deionized at 4°, frozen for 6 weeks; analyzed after standing 5 days at 25° at *p*H 3.0. (In all cases, sedimentation proceeds from right to left.)

only if the aggregation were mostly of a side-by-side rather than end-to-end type. The difference in the extent of aggregation observed with these two similarly treated solutions is a good example of the observed variation[17] in the extent and rate of aggregation of BSA in acid *p*H.

Another set of light scattering measurements was carried out with a BSA solution which had been deionized at 4°. In this case, light scattering measurements, reported in detail elsewhere,[13] were carried out in the *p*H range of 2.6–3.2 in HCl–KCl media of 0.01 and 0.20 ionic strengths. The solutions were measured both immediately after deionization and also after six weeks storage in a frozen condition. The deionized protein was adjusted to the conditions of the experiment and then permitted to stand overnight at refrigerator temperature. Each solution was then brought to 25°, filtered and used immediately in 90° light scattering measurements as a function of concentration. In view of the complete symmetry obtained in the multiangle experiments described above, no dissymmetry measurements were made. The results obtained yielded molecular weight values of a magnitude similar to that normally obtained for BSA at isoionic *p*H's (72,000–80,000).[15,20] Ultracentrifugal analyses carried out on these solutions immediately after the light scattering measurements showed no differences from pictures obtained at *p*H 5.6 in 0.1 *M* KCl.

When the solutions used in the last light scatter-

(20) S. N. Timasheff, H. M. Dintzis, J. G. Kirkwood and B. D. Coleman, *Proc. Nat. Acad. Sci. U. S.*, **41**, 710 (1955).

ing experiments were permitted to stand for five days at 25°, no change was observed in the ultracentrifugal patterns in the case of the 0.01 ionic strength solutions. In the case of the 0.20 ionic strength solutions, however, various degrees of aggregation were found, depending on the treatment of the deionized stock BSA solution. Thus, when the experiments were done on a freshly deionized BSA solution, the ultracentrifugal analyses, taken after five days at 25° followed by 21 days at 4° showed the presence of only 18% of the "dimer" (Fig. 3b), but no heavier (8.97 *S*) component. The deionized solution that had been kept for six weeks in a frozen condition prior to adjustment of the *p*H to 3.0, after standing for four days at room temperature in *p*H 3.0 KCl–HCl of 0.20 ionic strength, yielded ultracentrifugal pictures which showed the presence of equal amounts (*ca.* 15%) of the "dimer" and of the heavier (8.97 *S*) component (Fig. 3c).

These results, which indicate that aggregation occurs in BSA at *p*H 3.0 at high ionic strength, are in good agreement with recent reports.[4,5] The observed dependence of the aggregation on pretreatment of the BSA is also consistent with the findings of Halwer, *et al.*[9] Furthermore, the low values of the sedimentation constants of BSA obtained at *p*H 3 are in complete agreement with those reported by Bro, Singer and Sturtevant[5] and, as proposed by these authors, they "provide additional evidence" for the reversible expansion of BSA in acid solution, described by Yang and Foster[21] and by Tanford, *et al.*[22]

It would appear from the above observations that deionization in the cold followed by immediate use of the protein solution results in a preparation which is fairly resistant to aggregation at acid *p*H's. Freezing of the deionized solution or deionization at room temperature followed by lyophilization results in a more labile material. The data obtained in the *p*H region of 4.0 to 5.0 with deionized and non-deionized preparations indicate that in the latter case complications arise due to the presence of an ionic "impurity." This leads to the formation of very heavy aggregates which, however, can be dissociated by dilution. Since the dilution also reduces the concentration of the free "impurity" in the solution, it might possibly lead to a shift in the equilibrium toward disaggregation. That the size of the aggregate in the last case is very large and its concentration is small can be concluded from the pronounced dissymmetry of light scattering and also from the fact that ultracentrifugal analyses of BSA under similar conditions give no evidence for the presence of significant amounts of very large components.

(21) T. J. Yang and J. F. Foster, *J. Am. Chem. Soc.*, **76**, 1588 (1954).
(22) C. Tanford, J. G. Buzzell, D. G. Rands and S. A. Swanson, *ibid.*, **77**, 6421 (1955).

Summary.—Light scattering and ultracentrifugal measurements have been carried out on deionized and non-deionized solutions of BSA in acid *p*H's. It was found that while the non-deionized solutions aggregated readily, the deionized protein aggregated only at the lower *p*H's (~3) in high salt concentration. The rate and extent of aggregation were erratic. No appreciable dissymmetry was observed with the deionized protein, indicating that the associated units are compact.

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